Menstrual Blood Human Papillomavirus16 DNA Quantitation in Patients with Cervical Intraepithelial Neoplasia or Condyloma Acuminatum

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Objective: To quantitate the human papillomavirus16 (HPV16) DNA copy numbers in menstrual blood of patients with cervical intraepithelial neoplasia and condyloma acuminatum.
Methods: HPV16 DNA–positive menstrual blood specimens were collected from 27 patients with cervical intraepithelial neoplasia or condyloma acuminatum, who attended the Department of Obstetrics and Gynaecology, Queen Elizabeth Hospital, Hong Kong from 2007 to 2009. Two menstrual blood HPV16 DNA–positive sexually active apparently normal subjects were also included as controls. Primers and Taqman minor groove binder probes were designed and validated before using them to measure the HPV16 DNA copy number in menstrual blood specimens. Moreover, the median menstrual blood β-actin copy numbers from 29 menstrual blood HPV16 DNA–positive specimens were used to normalise the corresponding menstrual blood HPV16 DNA copy numbers from the same batch of specimens.
Results: The normalised menstrual blood HPV16 DNA copy numbers were significantly increased from apparently normal subjects to cervical intraepithelial neoplasia 3 ($p<0.0001$, Spearman rank correlation test).
Conclusion: Normalised menstrual blood HPV16 DNA copy numbers are a potential non-invasive marker of high-grade cervical intraepithelial neoplasia.
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Keywords: Cervical intraepithelial neoplasia; Condyloma acuminatum; DNA, viral; Menstruation; Papillomavirus infections

Introduction

Our previous study has shown that menstrual blood (MB) human papillomavirus (HPV) DNA is a potential non-invasive marker for pre-malignant cervical diseases, because MB HPV DNA was detected and genotyped in 83% of patients with cervical intraepithelial neoplasia (CIN) or condyloma acuminatum (CAC), 22% of patients with high-grade (HG) CIN after the loop electrosurgical excision procedure treatment and 0% of patients with low-grade (LG) CIN or CAC in complete remission[1]. Some reports have shown that HPV16 DNA concentration may be a predictor of the risk of developing HG CIN[2,3]. In this study,

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therefore, we measured the HPV16 DNA viral load using quantitative real time–polymerase chain reaction (QRT-PCR) in HPV16 DNA–positive MB specimens. These were detected using conventional PCR and the presence of HPV16 DNA was confirmed by direct sequencing[1]. The results could be important for understanding the potential of using HPV16 DNA copy numbers as a non-invasive marker for HG CIN.

Methods

Patient Samples

Twenty-nine HPV16 DNA–positive MB samples were used from our previous study[1]. Among the corresponding patients, the distribution of pathological stages were: CAC (4), CIN 1 (6), CIN 2 (8), and CIN 3 (9). The 27 patients (age range, 22 to 41; mean, 31 years) were recruited before treatment or remission in the Department of Obstetrics and Gynaecology, Queen Elizabeth Hospital, Hong Kong Special Administrative Region between 2007 and 2009. CIN is the pre-malignant lesion which is divided into HG CIN (CIN 3 and CIN 2) and LG CIN (CIN 1), whereas CAC is the benign lesion in the cervix[4]. Finally, two sexually active apparently normal subjects (ANS) (aged 27 and 30 years; mean, 29 years), who did not have any prior cervical disease were also found to be MB HPV16 DNA–positive and recruited as control. All participants were subjected to a short interview that included a brief introduction to the study, and questions concerning their medical, gynaecological, and sexual history. The study protocol was approved by the Clinical Research Ethics Committee, Queen Elizabeth Hospital, Hong Kong Special Administrative Region. Written consent was obtained from all participants.

Menstrual Blood HPV16 DNA Measurement Using Quantitative Real Time–Polymerase Chain Reactions

Each MB sample was collected in a sanitary napkin, put inside a ziplock plastic bag and sent to the laboratory by mail or by hand delivery. A small piece of sanitary napkin was cut out (1.5 cm x 1.5 cm x 5 mm) using sterile scissors. Genomic DNA was extracted from the MB on it, using the commercial QIAamp DNA Mini Kit (Cat. no. 51306, Qiagen, Hilden, Germany) according to the dried blood spot protocol. To measure MB HPV16 DNA copy numbers, primers and Taqman minor groove binder (MGB) probe, labelled with a carboxyfluorescein reporter dye at the 5'-end and a nonfluorescent quencher at the 3'-end, were designed at the E6 open reading frame (forward primers: 5'-CACCAAAAGAGAACTGCAATGTTT-3'; reverse primers: 5-TTTGCAGCTCTGTGCATAACTGT-3'; probe: 5'FAM-ACCCACAGGAGCGAC-3').

The specificity of the HPV16 DNA primers and probes were validated by quantifying MB HPV16 DNA from subjects with HPV16 infections (n = 29), subjects with HPV other than type 16 infections (n = 47) and subjects without HPV infection (n = 79). Results were positive for all HPV16 DNA–positive specimens and negative for others (data not shown). Therefore the primers and probes were specific for MB HPV16 DNA measurement. In order to quantify the amount of cellular DNA in the sample, MB β-actin DNA copy numbers were measured using Taqman primers and the MGB probe (401846; Applied Biosystems, Foster City, CA, USA). This was because β-actin DNA is a housekeeping gene which is present in every single nucleated cell and the median MB β-actin DNA copy numbers from 29 MB HPV16 DNA–positive patient specimens was used to normalise the HPV16 DNA copy numbers for the same batch of specimens. By this means, a fixed amount of cells in each MB specimen was used to measure the respective HPV16 DNA copy numbers.

QRT-PCR was performed in a reaction volume of 25 μl using Taqman Universal PCR Master Mix (4304437; Applied Biosystems), and 100 ng in 2.5 μl of extracted genomic DNA was used for each reaction. The standard protocol of the ABI Prism 7500 Sequence Detector (Applied Biosystems) was used. Absolute MB HPV16 and MB β-actin copy numbers of each patient sample were obtained using standard curves prepared by amplification of the respective plasmid-cloned HPV16 DNA and β-actin DNA at known input concentrations ranging from 0 to 1,000,000 copy numbers. Plasmids containing HPV16 and β-actin sequences were prepared by cloning their PCR products separately into the pCR2.1 vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The plasmids with integrated sequences were purified using QIAprep Spin Miniprep Kit (Qiagen, GmbH, D-40724 Hilden, Germany) also according to the manufacturer's instructions. Each batch of amplifications included multiple positive and negative controls. Duplicate tests were performed and the mean was calculated for each sample.

Statistical Analysis

Copy numbers were $\log_{10}$-transformed and summarised in scatter plots. The non-parametric Spearman rank correlation test was used to measure the correlation between normalised MB HPV16 DNA copy numbers and the stages of CIN.

Results

MB HPV16 DNA was confirmed by direct

sequencing to be positive in nine patients with CIN 3, eight with CIN 2, six with CIN 1, four with CAC, and two ANS. QRT-PCR showed that MB HPV16 DNA (Figure 1) and MB β-actin DNA (Figure 2, range of copy numbers: 16,995 to 158,745; median: 88,659) were positive in all 29 subjects, but with varying copy numbers. Normalising the absolute MB HPV DNA copy number of each subject using the corresponding MB β-actin DNA median copy numbers showed that the normalised MB HPV16 DNA copy numbers were significantly increased from ANS to CIN 3 ($p<0.0001$, Spearman rank correlation test). Furthermore, there was a significant difference in normalised MB HPV16 DNA copy number between patients with HG CIN and those without HG CIN ($p<0.0001$, Mann-Whitney test).

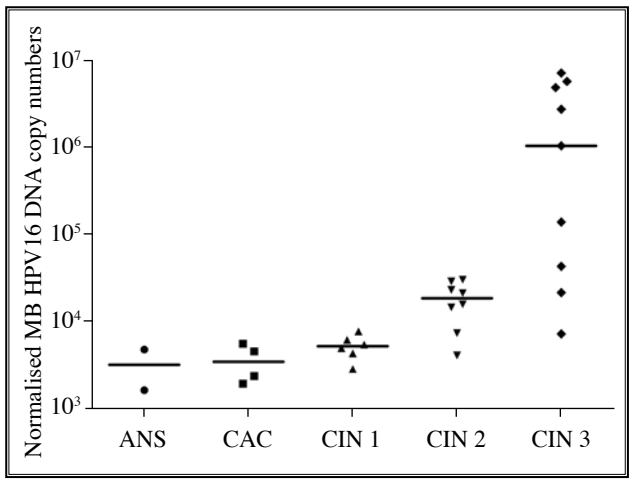

Figure 1. Normalised menstrual blood (MB) HPV16 DNA copy numbers in apparently normal subjects (ANS), patients with condyloma acuminatum (CAC), cervical intraepithelial neoplasia 1 (CIN1), CIN2, and CIN3

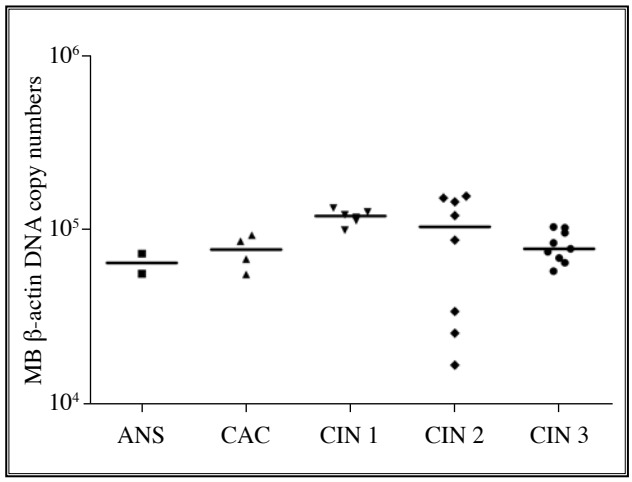

Figure 2. Menstrual blood (MB) β-actin DNA copy numbers in apparently normal subjects (ANS), patients with condyloma acuminatum (CAC), cervical intraepithelial neoplasia 1 (CIN1), CIN2, and CIN3

Discussion

MB provides a rich and stable source of materials for detecting HPV DNA. Compared to the Pap test, collection of MB is completely non-invasive. Moreover, women do not need to visit an outpatient clinic for sample collection, and by using a cytobrush both pain and embarrassment can be avoided.

The findings from this study show that MB HPV16 DNA copy numbers are a potential non-invasive marker for HG CIN because they were significantly increased in patients with CIN 3 (compared to ANS patients) and there was a significant difference in normalised MB HPV16 DNA copy numbers between patients with and without HG CIN. However, a few patients with CIN 3 had relatively low HPV16 DNA copy numbers, which may indicate a weak predictive value. We therefore need to validate our results using a larger cohort of HPV16 DNA–positive specimens. On the other hand, a broad range of MB β-actin DNA copy numbers for the 29 MB HPV16 DNA–positive subjects suggests that normalisation of MB HPV16 DNA results is essential and that the broad range of MB β-actin DNA copy numbers may be explained in two ways: (1) there could be varying degrees of cellular degradation in the MB specimens, and (2) β-actin DNA may not be an appropriate housekeeping gene for MB specimens. Therefore, standardisation of MB collection and delivery procedures is necessary to prevent inconsistency in DNA quality to overcome inaccurate HPV16 DNA quantitation. Despite this, our findings have important clinical implications because they show that the normalised MB HPV16 DNA viral load may be useful for follow-up of CIN patients for relapse after treatment or progression. Accordingly, a larger-scale study is being carried out to examine the prognostic significance of MB HPV16 DNA in patients with CIN.

To our knowledge, this report is the first to show that MB HPV16 DNA copy numbers is a potential non-invasive marker for HG CIN. This test can be applied to women who are reluctant to participate in cervical screening (due to pain and / or embarrassment). Inserting a tampon inside vagina to self-collect samples for HPV detection yields a sensitivity of 67 to 94%[5], but this method creates discomfort and the detection rate varies according to the time and depth of tampon insertion[6]. The use of MB can circumvent these limitations as the HPV DNA is released and collected in the sanitary napkin during menstruation. Moreover, self-collected cells from tampon or vaginal swab require liquid-based storage and transport media which are inflammable and hazardous[6], whereas MB collected in sanitary napkin can be stored inside a ziplock bag for transportation. Another study by Tong et al[7] detected HPV DNA in 100% (17/17) of patients with CIN or CAC using

vaginal discharge collected on sanitary napkins. For HPV DNA detection, compared to vaginal discharge, using MB has an advantage because it can be collected monthly (i.e. regularly) in premenopausal women. The success in the quantitation of HPV16 DNA copy numbers using QRT-PCR has demonstrated the feasibility of measuring the copy numbers of other infectious agents such as *Chlamydia trachomatis*, Herpes simplex virus in dried MB specimens. Despite the significant improvements in HPV detection, genotyping and quantitation, three important issues have to be resolved before applying this test for large-scale screening. First, there should be a better way to store and transport samples to collection centres. By this means, sample degradation can be kept to a minimum and safety ensured during transport and disposal. Second, high-throughput processing (using automatic cutting machine to process MB specimens and automatic extraction workstation) is needed to streamline the workflow for DNA extraction. Third, there should be standardisation of extraction and detection protocols with the same sequence of primers and probes to process the MB specimens for HPV16 DNA quantitation. Hopefully, such measures could facilitate development of a cost-effective, non-invasive test to quantitate the MB HPV16 DNA copy numbers, which could be used as a prognostic marker for HG CIN.

Conclusion

This study showed that MB HPV16 DNA is a potential non-invasive marker for HG CIN.

Acknowledgements

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